THE LANCET Neurology

Supplementary webappendix

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APPENDIX
Supplemental information

Index case 1

This 16 year-old girl presented to the hospital with a four-day history of severe fatigue and headache, accompanied by vertigo, nausea, and scintillating scotomas. She complained of several months of memory difficulties, cognitive dysfunction, anxiety, depressed mood and fatigue. Her past medical history was significant for Hodgkin's lymphoma which was in remission since completing chemotherapy and radiation 10 months earlier. On the fifth day of admission, she had a generalized tonic-clonic seizure and rapidly progressed to having frequent seizures. Complete blood cell count, C-reactive protein and erythrocyte sedimentation rate were normal. Testing for anti-thyroid peroxidase, anti-thyroglobulin, anti-nuclear antibodies, anti-neutrophil cytoplasmic antibodies, and paraneoplastic antibodies (Hu, Ri, Yo, CRMP5, amphiphysin) were negative. Brain MRI on day 3 demonstrated multiple foci of increased T2/FLAIR signal in both hemispheres (Figure 4A, E). CSF analysis showed normal opening pressure, 23 white blood cells (WBC)/ mm3 (69% lymphocytes), protein concentration 60 mg/dL,normal glucose concentration and negative cytology. Gram stain, routine cultures and PCR testing for Herpes simplex virus, Enterovirus and *Mycoplasma pneumoniae* were negative. Serology for Cytomegalovirus, Epstein-Barr virus, Arbovirus, *Bartonella henselae*, and Lyme disease were negative.
Treatment with high-dose methylprednisolone was initiated on day 7. Very high doses of phenobarbital were required to suppress electrographic seizures. A subsequent course of plasma exchange on alternating days for one week failed to improve the seizure pattern. On day 10, repeat brain MRI showed increase of the size of the FLAIR/T2 abnormalities, mainly in the left temporal lobe, and multifocal new cortical and subcortical lesions in both cerebral hemispheres (Figure 4B, F). Brain biopsy on day 14 demonstrated intense diffuse reactive astrocytic gliosis throughout the cortex associated with microglial activation and a population of reactive T lymphocytes. Several days later, antibodies against unknown neuronal cell-surface antigens were identified in her CSF. She received high-dose corticosteroids, intravenous immunoglobulin, rituximab and cyclophosphamide. Phenobarbital coma was continued for four months; during this time breakthrough clinical and electrographic seizures occurred if the phenobarbital level was allowed to decrease. EEG recordings demonstrated generalized periodic discharges late in the first month of admission (Supplemental Figure 3 A).

After three months, the EEG showed more focal left-sided ictal activity. The phenobarbital dose was weaned and she began a slow neurological recovery with gradual resolution of the encephalopathic EEG pattern. Four months after admission a repeat lumbar puncture showed resolution of the leukocytosis; however, repeat MRI showed numerous new multifocal lesions throughout the brain with diffuse atrophy and moderate ex-vacuo ventricular dilatation (Figure 4 C, G). Six months after her initial presentation, she began to show more rapid neurological recovery. Repeat MRI demonstrated no new lesions, improvement or resolution of all previous lesions, and reduction of the previous seen diffuse atrophy (Figure 4 D, H). She was transferred to an inpatient rehabilitation facility seven months after presentation and over the subsequent three months made significant gains to the point that she was able to communicate, eat, dress and groom herself. She could walk short distances with minimal assistance. Ten months after symptom onset, she was discharged home able to carry out most activities of daily living independently. At the last follow-up, 15 months after symptom onset, she walks with no assistance and is able to perform all daily

activities independently. She has returned to school with a modified course load due to mild cognitive deficits that continue to improve.

Index case 2

A 51 year-old man was admitted to the hospital for rapidly progressive symptoms of change of behavior and new-onset psychosis. Prior to admission the patient was seen several times in the emergency department of another hospital where he was diagnosed with new onset depression and treated with sertraline and alprazolam. In addition, he had complained of generalized pruritis and developed worsening high blood pressure. On several occasions the family heard the patient saying he was going to kill other people and himself. A few days prior to admission, he refused to get out of bed, and became apathetic with almost total reduction of verbal output. His past medical history was relevant for high blood pressure, diabetes mellitus, hypercholesterolemia, stroke (from which he had fully recovered), and thrombotic thrombocytopenic purpura treated a few years earlier with splenectomy and steroids.

At admission, the clinical picture resembled akinetic mutism, with brief periods in which the patient spontaneously uttered a few incoherent sentences. The day of admission, he was noted to have clonic seizures involving the left side of the face and left arm that resolved with intravenous diazepam and levetiracetam. Over the next 24 hours he developed acute respiratory failure due to pneumonia, requiring intubation and admission to intensive care unit. Two days later he developed status epilepticus characterized by clonic movements of the left side of the face and left arm, associated with continuous saccadic eye movements to the left that were refractory to all treatments, including levetiracetam, lacosamide, and phenytoin. The patient was maintained in a pharmacological coma, sequentially using midazolam, propofol, and thiopental. The seizures persisted until the patients' death 10 weeks after presentation.

The initial EEG showed seizures in the right temporal lobe with a tendency to generalization that in subsequent recordings progressed to a pattern of generalized periodic discharges (Supplemental Figure 3 B). The MRI showed multiple increased FLAIR/T2 signal abnormalities, extensively involving cortex without mass effect or contrast enhancement, blurring the grey-white matter junction (Figure 5). The initial CSF study was normal, but a repeat CSF analysis several days later showed IgG and IgM oligoclonal bands without matching serum bands. The following tests were negative: 1) Blood studies for syphilis, hepatitis virus B and C, *Brucella melitensis*, *Borrelia burgdorferi*, *Toxoplasma gondii*, *Streptococcus pneumoniae*, and *Legionella pneumophila*; 2) CSF studies for bacterial and fungal infections, Herpes simplex virus 1 and 2; Human herpesvirus 6, Cytomegalovirus, Varicella zoster virus, JC virus and Enterovirus, 3) panel for paraneoplastic antibodies, and connective tissue disorders (antibodies to GAD65, Hu, Ri, Yo, CRMP5, amphiphysin, DNAdc, Sm, Rib-P, PCNA, U1-RNP, SS-A/Ro, SS-B/La, Scl-70, CENP-B, RNA Pol III, Jo-1, Mi-2, PM-Scl, and ANCA), complement levels, 4) serum protein electrophoresis, 5) tumor markers: CEA, AFP, Ca 19.9, PSA, and B-2-microglobulina. The patient was found to have low levels of thyroid peroxidase antibodies (156 IU/ml) and thyroglobulin antibodies (158 IU/ml).

After excluding an infectious etiology, the patient was started on corticosteroids and IVIG without significant effect. One week later, he received 5 plasma exchange treatments without clinical effect and no change in the MRI (Figure 5 D-F). By this time laboratory studies revealed serum and CSF antibodies against unknown neuronal cell-surface antigens, and he was started on cyclophosphamide (1 g per m^2/ month) and

rituximab (1 g every 2 weeks). Despite these treatments the patient showed no clinical or radiological improvement and continued with electrographic status epilepticus. Repeat MRIs showed new FLAIR/T2 abnormalities diffusely involving cortex (Figure 5 G-I), and the patient died two months after admission.

Supplemental methods

Immunohistochemistry of rat brain

Adult female Wistar rats were sacrificed without perfusion, and the brain was removed and fixed by immersion in 4% paraformaldehyde for 1 hour at 4°C, cryoprotected in 40% sucrose for 48 hours, embedded in freezing compound media, and snap frozen in isopentane chilled with liquid nitrogen. Seven-micrometer-thick tissue sections were then sequentially incubated with 0.3% H_2O_2 for 15 minutes, 5% goat serum for 1 hour, and patient or control serum (1:200), or CSF (1:5) at 4°C overnight. After using a secondary biotinylated antibody goat anti-human IgG (diluted 1:2000, Vector, BA-3000), the reactivity was developed with the avidin-biotin-peroxidase method, as reported.[1]

Immunocytochemistry on neuronal cultures

Rat hippocampal neuronal cultures were prepared as reported.[2] Live neurons grown on coverslips were incubated for 1 hour at 37°C with patient or control serum (final dilution 1:200) or CSF (1:10). After removing the media and extensive washing with phosphate-buffered saline (PBS), neurons were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and immunolabeled with Alexa Fluor 488 goat anti-human IgG (diluted 1:1000, Invitrogen, A11013). Results were photographed under a fluorescence microscope using Zeiss Axiovision software (Zeiss, Thornwood, NY).

Immunocytochemistry on HEK293 cells

Fixed cells:

HEK293 cells were transfected with plasmids containing the human α1 subunit of the GABAaR (accession number: NM_000806.3; Origene catalog number: SC119668) or the human β3 subunit of the receptor (accession number: NM_000814.3; Origene catalog number: SC125324); cells transfected with a plasmid without insert was used as control. Cells were grown for 24 hours after transfection before assessment. Transfected cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and then incubated with patients' serum (1:20 and higher serial dilutions) or CSF (1:5 and higher serial dilutions) along with a commercial mouse antibody against the α1 subunit of the GABAaR (dilution 1:5000, Millipore, MAB339) or the β3 subunit (dilution 1:5000, Abcam, AB4046) for 2 hours at room temperature, and the corresponding fluorescent secondary antibodies (Alexa Fluor 488 goat anti-human IgG, diluted 1:1000, A11013; and Alexa Fluor 594 goat anti-mouse IgG, diluted 1:1000, A11032, both from Invitrogen). Results were photographed under a fluorescence microscope using Zeiss Axiovision software.

Live cells:

Live HEK cells were incubated with serum (1:20 and higher serial dilutions) or CSF (1:5 and higher serial dilutions) of the patient together with the same commercial antibody against GABAaR indicated above for 1 hour at 37ºC, washed, and fixed with 4% paraformaldehyde for 5 minutes. After washing cells were then incubated with the corresponding Alexa Fluor secondary antibodies indicated above.

Immunoprecipitation and immunoblot

Live neurons obtained as above, were grown in 100 mm plates (density 1.5 x 10^6 neurons/plate), and incubated at 37°C with filtered patient serum (diluted 1:200) for 1 hour. Neurons were then washed with PBS, lysed with buffer (NaCl 150mM, EDTA 1mM, tris (hydroxymethyl) aminomethane [Tris]-HCl 100mM, deoxycholate acid 0.5%, 1% Triton X-100, pH 7.5) containing protease inhibitors (P8340; Sigma Labs), and centrifuged at 16.1 x 10^3 *g* for 20 minutes at 4°C. The supernatant was retained and incubated with protein A/G agarose beads (20423; Pierce, Rockford, IL) overnight at 4°C, centrifuged, and the pellet containing the beads with patients' antibodies bound to the target cell-surface antigen was then washed with lysis buffer, aliquoted, and kept at -80°C. An aliquot of this pellet was resuspended in Laemmli buffer, boiled for 5 minutes, separated in a 4 to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the proteins visualized with EZBlue gel staining (G1041; Sigma Labs). Due to the lack of differences between the EZBlue-visible bands between patient's and control samples, all precipitated proteins run along the gel were analyzed using mass spectrometry.

Mass spectrometry

Mass spectrometry was performed at the Proteomics Facility at the Abramson Cancer Center of the University of Pennsylvania. Protein bands were trypsin digested and analyzed with a nano liquid chromatography (nano LC)/nanospray/linear ion trap (LTQ) mass spectrometer (Thermo Electron Corporation, San Jose, CA) as reported.[3] Briefly, 3 ml trypsin digested sample was injected with autosampler from Eksigent (Dublin, CA). The digested samples were separated on a 10 cm C18 column, using nano LC from Eksigent with 200 ml/minute flow rate, 45 minute gradient. Online nanospray was used to spray the separated peptides into LTQ, and Xcalibur software (Thermo Scientific, Waltham, MA) was utilized to acquire the raw data. The raw data files were searched using Mascot (Matrix Science, Boston, MA) against the NCBI and Swissprot databases (Swiss Institute of Bioinformatics (Basel, Switzerland).

Immunoabsorption and immunocompetition studies

In order to determine whether the brain reactivity of patient's antibodies was specifically due to GABAaR binding, six 60 mm plates of HEK 293 cells expressing GABAaR were sequentially incubated with patient's serum (1:200), each plate for 1 hour at 37°C. After incubation with the six plates, the immunoabsorbed serum was incubated with sections of rat hippocampus, as above. Patient's serum absorbed with non-transfected HEK 293 cells served as control.

To determine whether patients' antibodies were directed against similar antigens and epitopes of GABAaR, immunocompetition studies were performed. IgG was isolated from a patient whose serum contained high levels of IgG antibodies against GABAaR using protein A and G sepharose beads, and subsequently eluted and labeled with biotin (Vector, SP1200), as reported.[4] Then, sections of rat brain were incubated with other patients' or control sera (diluted 1:5) overnight at 4^{o}C, washed in PBS, and subsequently incubated with the indicated human biotinylated IgG containing GABAaR antibodies (diluted 1:40) for 1 hour at room temperature, and the reactivity was developed using the avidin-biotin-peroxidase method. Two sera were considered to compete for the same GABAaR epitopes, when pre-incubation of the tissue with one serum abrogated the reactivity of the other patient's IgG.

Quantitative analysis of neuronal GABAaR immunolabeling by patient's antibodies

To determine the degree of immunolabeling of GABAaRs by patient's antibodies, 14-day *in vitro* (div) rat hippocampal neurons were incubated with a representative patient's CSF (diluted 1:20) for 30 minutes, then washed, fixed, and incubated with a commercial mouse monoclonal antibody (Millipore 05-474; 1:500) against a sequence contained in the β2/3 subunit (which is a component of most GABAaR[5]) followed by appropriate fluorescent-conjugated secondary antibodies, Alexa Fluor 488 goat anti-human IgG (1:200, Invitrogen, A11013) and Alexa Fluor 594 donkey anti-mouse IgG (1:200, A21203, both from Invitrogen). Images were obtained with a laser-scanning confocal microscope (Leica TCS SP5). Laser light levels and detector gain and offset were adjusted in every experiment so that no pixel values were saturated in any treatment conditions. Images were thresholded, and the number of individual clusters along neuronal dendrites was determined using interactive software (ImageJ).

Analysis of the structural effects of patient's antibodies on GABAaR clusters

To determine the effects of patient's antibodies on the number and localization of GABAaR clusters, 14 *div* rat hippocampal neurons were treated with patient's or control CSF (1:20 dilution in Neuro-Basal supplemented with B27 medium; GIBCO, Carlsbad, CA) for 2 days. Every day, 20 of the 300 µl medium in each culture well were removed and replaced with 20 µl fresh patient or control CSF. On 16 *div*, neurons were fixed in freshly made paraformaldehyde (4% paraformaldehyde, 4% sucrose in phosphate-buffered saline) for 5 minutes, permeabilized in 0.25% Triton X-100 for 10 minutes, and blocked in 5% normal goat serum for 1 hour. Neurons were then incubated with the indicated monoclonal antibody against the GABAaR β2/3 (1:500), or a mouse monoclonal antibody against Gephyrin (1:200, Synaptic Systems, 147011), or a guinea pig polyclonal antibody against vesicular-GABA transporter (VGAT, 1:1000; Synaptic Systems,131004) or a rabbit antibody against GluN1 (anti-NMDAR1, 1:100; Millipore AB9864R) for 2 hours, followed by the appropriate fluorescent-conjugated secondary antibodies (Alexa Fluor 488 goat anti mouse IgG, 1:200, A-11001; Alexa Fluor 594 goat anti-guinea pig IgG, A-11076, 1:200; Cy5 donkey anti-rabbit IgG, 1:200, Jackson ImmunoResearch 711-175-152). Images were obtained and analyzed as above.

Supplemental Table 1: Sequences isolated by immunoprecipitation with patient's serum

Sequence	β3 subunit of the GABAaR, peptide identification probability	Sequest XCorr	Sequest deltaCn
(R)LHPDGTVLYGLR(I) (+3H)	95%	2.97	0.21
R)NVVFATGAYPR(L) (+2H)	95%	3.21	0.55
(R)VADqLWVPDTYFLnDKK(S) (+3H)	95%	2.98	0.42

Mass spectral data was analyzed using the search engine Sequest. Peptide confidence was determined by the cross-correlation scoring which represent sensitivity, comparing the experimental fragmentation spectrum of the peptides against the theoretical predicted fragmentation spectrum; and by the DeltaCn, which represents specificity for the peptide identification. Xcorr > 2 (+2 H), 2.5 (+3 H) and deltaCn > 0.2) indicate a good spectrum.

Legends to Supplemental figures

Supplemental Figure 1: Comparison of reactivity of CSF of a patient with GABAaR antibodies with that of a patient with GABAbR antibodies using rat brain immunohistochemistry (high magnification of figure 1)
Panel A shows the reactivity of the CSF (dilution 1:4) of patient #2 with hippocampus; the asterisk indicates the area shown at higher magnification in panel B. Panel C shows the reactivity with cerebellum. Panels D-F correspond to the same brain regions immunostained with CSF of a patient with GABAbR antibodies, and panels G-I with CSF of a control subject (without GABAaR or GABAbR antibodies). Note that the pattern of reactivity of GABAaR antibodies is very similar to that of the GABAbR antibodies which makes difficult to distinguish one from the other by plain immunohistochemistry. Scale bar for G = 100 μm, Scale bar for H and I = 200 μm

Supplemental Figure 2: Immunocompetition studies demonstrating that patients' antibodies compete for the same epitopes of the GABAaR
Reactivity with rat brain of biotinylated IgG from a patient with GABAaR antibodies in which the tissue has been pre-incubated with serum from a control individual (A and B), the serum from the same patient whose IgG has been biotinylated (C, D), and the serum of another patient with GABAaR antibodies. Note the dramatic decrease of reactivity (competition for the same GABAaR epitopes) in panels E and F compared with A and B. Panels C and D (competition with same patient's serum serves to demonstrate the background reactivity). Scale bar for A, C, E = 1 mm; Scale bar for B, DD and E = 200 μm

Supplemental Figure 3: Generalized periodic discharges in patients with encephalitis and antibodies to GABAaR

The recording in A corresponds to the EEG of patient #1 obtained one month after admission; note the presence of generalized epileptiform discharges. Settings: gain (sensitivity): 5 μV/mm; low frequency filter: 1 Hz, high frequency filter: 70 Hz.
The recording in B corresponds to patient #2; this patient initially showed epileptiform activity in the right temporal lobe with tendency to generalization in posterior recordings, as shown in B. Settings: gain (sensitivity): 15 μV/mm; low frequency filter: 0.5 Hz, high frequency filter: 35 Hz.

Supplemental Reference List

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